IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant:	Ole SIBBESEN *et al.*	**Filed via EFS Web March 27, 2009**
Title:	BACTERIAL XYLANASES	
Appl. No.:	10/626,583	
Filing Date:	7/25/2003	
Examiner:	Ganapathiram RAGHU	
Art Unit:	1652	
Confirmation Number:	9539	

BRIEF ON APPEAL

Mail Stop **Appeal Brief - Patents**
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Under the provisions of 37 C.F.R. § 41.37, this Appeal Brief is being filed together with a credit card payment form in the amount of $540.00 covering the 37 C.F.R. 41.20(b)(2) appeal fee. If this fee is deemed to be insufficient, authorization is hereby given to charge any deficiency (or credit any balance) to the undersigned deposit account 19-0741.

I. REAL PARTY IN INTEREST

The real party in interest is Danisco A/S as reflected on an assignment recorded on October 1, 2001 at Reel 012213, Frame 0695 in parent U.S. Application No. 09/869,155. The present application is a continuation application of U.S. Application No. 09/869,155.

II. RELATED APPEALS AND INTERFERENCES

No other prior or pending Appeals, interferences or judicial proceedings are known to Appellants or the Appellants' legal representatives which may be related to, directly affect or be directly affected by, or have a bearing on a decision by the Board of Patent Appeals and Interferences ("the Board") in the present Appeal.

III. STATUS OF CLAIMS

Claims 56-67 and 69-70 are pending in the present application.

Claims 1-9, 11, 12, 14-43, 48-55 and 68 have been cancelled.

Claims 13 and 44-47 are withdrawn.

Claims 56-67 and 69-70 are rejected.

Claims 56-67 and 69-70 are appealed.

IV. STATUS OF AMENDMENTS

No amendments to the claims have been filed subsequent to the Final Office Action dated July 31, 2008.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Claims 56 and 60 are the sole independent claims on appeal. Claim 56 is directed to a bakery product or dough for making a bakery product, suitable for use in a foodstuff, comprising

a polypeptide which is expressed from the nucleotide sequence of SEQ ID NO:6. The polypeptide encoded by SEQ ID NO:6 is a bacterial xylanase isolated from *Bacillus subtilis*. Support for claim 56 can be found in the specification at ¶¶ [0131], [0134], [0146]-[0152], and the sequence listing, of U.S. Patent Application Publication No. 2004/0234998, which corresponds to the captioned application.

Claim 60 is directed to a dough for making a bakery product prepared by incorporating the bacterial xylanase expressed from the nucleotide sequence of SEQ ID NO:6, in which the resultant dough is less sticky than an otherwise identical dough prepared by incorporating a fungal xylanase instead of a bacterial xylanase. Support for clam 60 can be found in originally filed claims 44 and 48, as well as the specification at ¶¶ [0131], [0134], [0146]-[0152], and the sequence listing, of U.S. Patent Application Publication No. 2004/0234998

Claims 57-59 all depend directly or indirectly from claim 56 and recite further features of the polypeptide expressed from the nucleotide sequence of SEQ ID NO:6 such as the polypeptide does not contain a leader sequence or has the amino acid sequence of SEQ ID NO:5 or amino acids 29-213 of SEQ ID NO:5. Support for claim 57-59 can be found in the originally filed claims and ¶¶ [0172]-[0174] of U.S. Patent Application Publication No. 2004/0234998.

Claims 61-67 and 69-70 all depend directly or indirectly from claim 60 and recite further features of the polypeptide expressed from the nucleotide sequence of SEQ ID NO:6 (*i.e.*, the polypeptide does not contain a leader sequence or has the amino acid sequence of SEQ ID NO:5 or amino acids 29-213 of SEQ ID NO:5); or further features of the dough itself (*i.e.*, comprising wheat flour and water or yeast) or further features of the bacterial xylanase, *e.g.*, that it is free of detrimental levels of glucanase enzymes or from a *Bacillus subtilis* strain, or are directed to the bakery product prepared by baking the dough of claims 60 or 69, respectively. Support for claims 61-67 and 69-70 can be found in the originally filed claims and ¶¶ [0172]-[0174] of U.S. Patent Application Publication No. 2004/0234998.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issues on appeal are: (1) whether claims 56-66, 69 and 70 are obvious under 35 U.S.C. § 103(a) over U.S. Patent No. 5,176,927 to Haarasilta *et al.* ("Haarasilta") or Poutanen, K., *Trends in Food Science and Techol.* 8:300-306 (1997) (Poutanen) in view of Paice *et al.*, *Arch. Microbiol.* 144:201-206 (1986) and Accession No. P184229, UnitProt Database 1990 ("Paice") or Wolf *et al.*, Accession No. I40569, PIR Database, 1996 and *Microbiology* 141:281-290 (1995) ("Wolf") and U.S. Patent No. 5,405,769 to Campbell *et al.* ("Campbell") and (2) whether claim 67 is obvious over Haarasilta or Poutanen, Paice or Wolf and Campbell and further in view of Autio *et al.*, Academic Press, 18-27 (1996) ("Autio").

VII. ARGUMENT

1. Claims 56-66, 69 and 70 are not obvious over the cited art.

In proceedings before the Patent and Trademark Office, the Examiner bears the burden of establishing a *prima facie* case of obviousness based upon the prior art. *See In re Piasecki,* 745 F.2d 1468, 1471-73 (Fed. Cir. 1984). As set forth in *Graham v. John Deere Co. of Kansas City*, "[u]nder § 103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is to be determined." 383 U.S. 1, 17 (1966). This has been the standard for 40 years, and remains the law today. *See KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727 (2007). If, after these criteria are considered, the evidence indicates that the claimed invention is obvious over the prior art, it may be said that a *prima facie* case of obviousness has been established.

In addition, the Examiner must show reasons, explicit or otherwise, that would compel one of ordinary skill in the art to combine the references to make and use the claimed invention. To determine whether there is "an apparent reason to combine" the known elements in the way an application claims,

> It will be necessary...to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art.... To facilitate review, this analysis should be made explicit.

Id. at 14; *See also* Memorandum from the United States Patent and Trademark Office, "Supreme Court decision on *KSR Int'l Co. v. Teleflex, Inc.*," (May 3, 2007) ("The Court did not totally reject the use of 'teaching, suggestion, motivation' as a factor in the obviousness analysis.... [I]n formulating a rejection...based upon a combination of prior art elements, it remains necessary to identify the reason why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed."). Moreover, reliance upon hindsight reasoning, based upon the Appellants' disclosure, in combining the cited references in support of the rejection under 35 U.S.C. § 103(a) is improper. *See KSR* at 17 ("A factfinder should be aware...of the distortion caused by hindsight bias and must be cautious of arguments reliant upon *ex post* reasoning."); *see also* MPEP § 2142.

A. The Examiner has failed to make a *prima facie* showing of obviousness under the rationale of *KSR*.

The Examiner alleges that the cited references render claims 56-67, 69 and 70 *prima facie* obvious to one of ordinary skill in the art when one applies the Teaching, Suggestion and Motivation test under the following rationale for arriving at a "conclusion of obviousness as suggested by the KSR ruling":

> (1) Combining prior art elements according to known method to yield predictable results.
>
> (2) Simple substitution of one known element for another to obtain predictable results.
>
> (3) "Obvious to try" – choosing from a finite number of identified, predictable solution [*sic*], with a reasonable expectation of success.

Advisory Action Dated October 20, 2008 ("Advisory Action") at 11-12 and Final Office Action dated July 31, 2008 ("Final Office Action") at 13.

Additionally, the Examiner alleges that his argument is based on "Graham factual enquiries [*sic*]":

> (1) a finding that at the time of the invention, there had been a recognized problem or need in the art, which may include a design need or market pressure to solve a problem; the need here is use of novel xylanases with desirable properties for use in the a [*sic*] bakery product or dough and to identify xylanases to achieve reactions that yield the optimal alteration in the molecular properties, microstructure, functional properties and product quality.
>
> (2) a finding that there had been a finite number of identified, predictable potential solutions to the recognized need or problem; Knowledge of use of xylanases in the baking industry is well known in the art.
>
> (3) a finding that one of ordinary skill in the art could have pursued the known potential solutions with a reasonable expectation of success; and therefore there is clear motivation for commercial exploitation;...Paice et al., Wolf et al., Campbell et al., and Haarasilta et al., teach the structural and functional elements of the instant invention i.e. an enzyme with endo-xylanase activity.

Advisory Action at 12-13 and Final Office Action at 13-14 (emphasis in original). The Examiner concludes that the present claims are thus *prima facie* obvious in that "one of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely that the product [was] not of innovation but of ordinary skill and common sense." Advisory Action at 13 and Final Office Action at 14 (emphasis and text in brackets in original).

(i). The present invention is not a combination of prior art elements according to known methods which yield predictable results.

The Examiner relies upon Haarasilta and Poutanen as the primary references in the present rejection. Haarasilta discloses the use of xylanases generally in baking products. However, Haarasilta does not teach or suggest the use of a bacterial xylanase as is presently

claimed. Poutanen discusses that the use of xylanases generally increases viscosity, results in better "ovenspring," increases bread volume, and decreases staling in bread. *See, e.g.*, Poutanen at 303, right column and 304, bottom left column to right column. The only reference to dough stickiness in Poutanen may be found in Table 3, on page 304. The Table contains a list of effects disclosed for certain xylanases together with a reference to the document which discloses such effects. All of the xylanase examples found in Table 3 are xylanases extracted from fungal sources, *i.e.*, *Aspergillus* and *Trichoderma*. The Table discloses that a specific fungal xylanase from *Aspergillus* caused an increase in the specific volume of wheat bread without causing stickiness:

> A specific *Aspergillus awamori* endo-xylanase was effective in increasing the specific volume of wheat bread without causing stickiness.

Poutanen at 304. Poutanen is silent with regard to the effect a bacterial xylanase, as is presently claimed, may have on the stickiness of the dough.

At the time of the claimed invention, bacterial xylanases were known to produce very sticky doughs, as discussed in the specification at paragraphs [0010] - [0012] of U.S. Patent Application Publication No. 2004/0234998. Additionally, references such as Maat *et al.*, "Xylanases and their application in bakery," in XYLANS & XYLANASES ed. J. Visser *et al.* Elsevier:349-360 (1992) ("Maat") (cited by Poutanen and cited as document **A27** in the IDS filed August 18, 2003) disclose that xylanases derived from fungal sources other than *Aspergillus awamori* and bacterial sources result in sticky dough:

> We have identified a particular β-(1,4)-xylanase produced by an Aspergillus niger var awamori strain as being very effective in increasing the specific volume of breads, without giving rise to a negative side effect on dough handling (stickiness of the dough) *as can be observed with xylanases derived from other fungal or bacterial sources*."

Maat at 349 (emphasis added).

The Examiner relies upon Paice or Wolf as allegedly describing a polynucleotide and polypeptide encoded by the polynucleotide having 100% sequence homology to the nucleotide sequence of SEQ ID NO:6 and the polypeptide of SEQ ID NO:5. Advisory Action at 4 and Final Office Action at 5. Paice relates to the isolation of a xylanase gene from *B. subtilis* and Wolf relates to the isolation of a xylanase gene from *B. subtilis* 168. Neither Paice nor Wolf disclose or suggest a bakery product or a dough for making a bakery product, let alone a bakery product or dough for making a bakery product, suitable for a foodstuff, comprising a polypeptide expressed from the nucleotide sequence of SEQ ID NO:6, as is presently claimed.

The Examiner also cites Campbell as allegedly describing a *Bacillus* xylanase enzyme having 100% sequence homology to the amino acid sequence of amino acid residues 29-213 of SEQ ID NO:5 (SEQ ID NO:37 of Campbell) and "also suggest[s] the use of said xylanase for altering the texture in bakery products (column 1)." Advisory Action at 4 and Final Office Action at 5. Campbell is directed to "a modified xylanase, which shows an improved thermostability when compared to the naturally occurring xylanase." Campbell at col. 1, ll. 7-9. Campbell is further directed to "a modified xylanase, wherein said xylanase has increased thermostability and wherein said xylanase is modified through either the introduction of a non-native disulfide bridge, introduction of an N-terminal mutation, or both." Campbell at col. 1, ll. 10-14. As such, Campbell is directed to modified xylanases, in particular modified xylanases of *B. circulans*, for use in the pulp manufacturing industry. *See* Campbell at Abstract. Campbell further mentions that thermotolerant variants of xylanases can also be used in the food processing industry. *Id.*

Campbell makes reference to a bakery use for xylanases in general, in the background section which lists uses in "non-pulp applications":

> Xylanase also has uses in non-pulp applications. Xylanases have been reported to be useful in clarifying juice and wine (Zeikus. J. G., Lee, Y.-E., and Saha, B. C. 1991. ACS Symp. Ser. 460:36-51; Beily, P. 1991. ACS Symp. Ser. 460:408-416; Woodward J. 1984. Top Enzyme Ferment. Biotechnol. 8:9-30), extracting coffee, plant

> oils and starch (McCleary, B. V. 1986. Int. J. Biol. Macromol. 8:349-354; Beily, P. 1991. ACS Symp. Ser. 460:408-416; Woodward J. 1984. Top Enzyme Ferment. Biotechnol. 8:9-30), for the production of food thickeners (Zeikus. J. G., Lee, Y.-E., and Saha, B. C. 1991. ACS Symp. Ser. 460:36-51), **altering texture in bakery products (Maat, J., Roza. M., Verbakel, J., Stam, H., Santos da Silva, M. J., Bosse, M., Egmond, M. R., Hagemans, M. L. D., v. Gorcom, R. F. M., Hessing, J. G. M., v.d. Hodel, C.A.M.J.J., and Rotterdam, C. 1992. In Xylans and xylanases. Visser, J., Beldman, G., Kusters-van Someren, M. A. and Voragen, A. G. J., eds. Elsevier Sci pub., Amsterdam. ISBN 0-444-894-772; McCleary, B. V. 1986. Int. J. Biol. Macromol. 8:349-354)**, and in the washing of super precision devices and semiconductors (Takayuki, I., Shoji, S. U.S. Pat. No. 5,078,802, issue date 92 Jan. 07). **Several of these application [*sic*] could benefit from a thermostable xylanase**, for example, food processing at elevated temperatures.

Campbell at col. 1, ll. 43-68 (emphasis added). Campbell cites Maat which teaches a fungal xylanase and, as explained *infra*, teaches away from the use of a bacterial xylanase in dough. Thus, Campbell teaches modified xylanases based on naturally occurring xylanases with a vague reference to their use in food processing. Thus, Appellants maintain that neither Paice, Wolf, nor Campbell teach or suggest that the claimed bacterial xylanase would have particular use in baking applications or doughs.

Appellants emphasize that one of skill in the art would not have been able to predict the effects on dough stickiness of a bacterial xylanase which is expressed from the nucleotide sequence of SEQ ID NO:6. Indeed, one of skill in the art would have predicted that a bacterial xylanase would have a *negative* impact on dough stickiness based on the teaching of Maat, for example. Furthermore, it was unexpected, and thus unpredictable, that the xylanase expressed from the nucleotide sequence of SEQ ID NO:6 would have resulted in decreased dough stickiness compared to other bacterial and fungal xylanases, as discussed further *infra*. None of the references teach or suggest that bacterial xylanases, and in particular the bacterial xylanase expressed from the nucleotide sequence of SEQ ID NO:6, would be effective in decreasing dough stickiness.

Thus, the present invention is not a combination of prior art elements according to known methods which yield predictable results or a simple substitution of one known element for another to obtain predictable results. One of skill in the art would not have predicted that a bacterial xylanase would be effective in decreasing dough stickiness.

(ii). Claimed invention is not "obvious to try."

Appellants also emphasize that the present invention cannot be considered "obvious to try" as a finite number of identified, predictable solutions did not exist. Furthermore, based on the unpredictability in the art described *supra*, one of skill in the art would not have had a reasonable expectation of success.

Indeed, there were a vast number of xylanases, from many different organisms, known at the time of the invention. A quick search of the NCBI protein database (www.ncbi.nlm.nih.gov/sites/entrez) for "xylanase" sequences published between January 1, 1980 and January 1, 1999 resulted in 494 sequences. One of skill in the art could have chosen any one of these xylanases. Furthermore, as discussed, *supra*, one of skill in the art would have had no reasonable expectation of success that bacterial xylanases would have improved certain qualities of dough or bakery products without the negative side-effect of dough stickiness.

The Examiner has provided no motivation as to why one of skill in the art would have used the specifically claimed bacterial xylanase obtained by expressing the polynucleotide sequence of SEQ ID NO:6 or the xylanase having amino acids 29-213 of SEQ ID NO:5 over all other xylanases which were known in the art, specifically since it was known in the art that bacterial xylanases were known to produce very sticky doughs. Specification at ¶¶ [0010] - [0012] and Maat.

This broad selection of xylanases, with no reasonable expectation of success, cannot form the basis for a finite number of "identified and predictable solutions" as required by the *KSR* and *Takeda Chemical Industries, Ltd. V. Alphapharm Pty., Ltd.*, 492 F.3d 1350, 1359 (Fed. Cir. 2007) (stating that "the prior art disclosed a broad selection of compounds any one of which

could have been selected as a lead compound for further investigation."). The court emphasized that obviousness requires that the prior art give a reason or motivation to make the specific composition claimed. *Id.* at 1356. No art has been cited which would have narrowed the choices of a xylanase for a dough with decreased stickiness. *Takeda*, 492 F.3d 1360 (invention was non-obvious as there was nothing in the prior art to narrow the possibilities of a lead compound to modify.). Here, where the record reveals no such reason for selecting the xylanase produced by the polynucleotide of SEQ ID NO:6, the obviousness rejection is improper, and should be reversed.

(iii). No motivation to select the bacterial xylanase produced by the polynucleotide of SEQ ID NO:6 without the use of impermissible hindsight.

The Examiner maintains that there would have been motivation to select the bacterial xylanases of Paice, Wolf or Campbell as one of skill in the art would have been interested in understanding the effect of the cited xylanases in a bakery product or dough:

> [T]hus a skilled artisan would certainly be interested to understand the effect of the xylanase isolated by Paice et al., or Wolf et al., or Campbell et al., on a bakery product or a dough, i.e. its mechanism of action and would be motivated to employ the xylanase isolated by Paice et al., or Wolf et al., and Campbell et al.

Advisory Action at 10 and Final Office Action at 11. However, the Examiner has failed to provide a motivation as to why one of skill in the art would have chosen the particular bacterial xylanase of Paice, Wolf or Campbell over all other xylanases known at the time of the invention. Relying upon impermissible hindsight, the Examiner has arrived at the xylanases disclosed in Paice, Wolf and Campbell based on sequence comparison from Appellants' disclosure. *See KSR* at 17 ("A factfinder should be aware...of the distortion caused by hindsight bias and must be cautious of arguments reliant upon *ex post* reasoning."); *see also* MPEP § 2142.

Here, at the time of the invention, a large number of xylanases were known, any of which could have been selected by one of skill in the art. The Examiner has not provided any reason

why out of the numerous known xylanases at the time of the invention, one of skill in the art would have chosen the bacterial xylanase produced by the polynucleotide of SEQ ID NO:6. Additionally, neither Paice, Wolf nor Campbell teach or suggest that the claimed xylanase would have use in particular baking applications. The Examiner has arrived at the xylanases disclosed in Paice, Wolf and Campbell based solely on hindsight sequence comparison from Appellants' disclosure.

Appellants maintain that the Examiner relies upon impermissible hindsight to create his rejection. Thus, the present invention cannot be obvious.

Therefore under *Graham* and *KSR*, the Examiner has failed to establish a *prima facie* case of obviousness as one of skill in the art would neither have predicted, nor had a reasonable expectation of success, that a bacterial xylanase would be effective in decreasing dough stickiness. Furthermore, a finite number of identified, predictable solutions did not exist as it would not have been obvious to select the bacterial xylanase encoded by the polynucleotide sequence of SEQ ID NO:6 from the vast array of known xylanase protein sequences. The Examiner relies solely upon impermissible hindsight to combine the cited references. Appellants maintain that the invention is, therefore, not rendered obvious in view of the cited art.

B. Appellants' Claimed Invention Demonstrates Unexpected Results.

The claimed invention demonstrates unexpected results which are indicia of nonobviousness and must be considered by the Examiner. MPEP § 716.01(a). The Examiner has failed to comment regarding Appellants' discovery that the xylanase produced by the polynucleotide sequence of SEQ ID NO:6 had a surprising and unexpected effect. In particular, the claimed xylanase produced a significantly less sticky dough as compared with other xylanases, including other bacterial xylanases. *See* Example 1.

In Example 1 of the present application, two fungal xylanases (X1 and Nova) are compared with the bacterial xylanase obtained by expression of the nucleotide sequence of SEQ

ID NO:6 (referred to as BX in the captioned application). *See e.g.* Tables 1 and 2 of the specification. Use of the bacterial BX xylanase resulted in dough that was less sticky than other fungal xylanases. Additionally, Example 1 of the specification compares the Röhm bacterial xylanase (which differs from the xylanase of the present invention by only a small number of amino acids) with the bacterial BX xylanase obtained by expression of the nucleotide sequence of SEQ ID NO:6. *See e.g.* Tables 3 and 4 of the specification. The results in the captioned application demonstrate that BX gave rise to a less sticky dough compared with the Röhm bacterial xylanase.

As discussed previously, at the time of the claimed invention, bacterial xylanases were known to produce very sticky doughs. Specification at ¶¶ [0010] - [0012] and Maat. Thus, it was unexpected that the use of the bacterial xylanase (BX) resulted in dough which was less sticky than other fungal xylanases. Furthermore, it was unexpected that another bacterial xylanase (Röhm), with a similar amino acid sequence, would not have the same effect.

These results also show that there was a high level of unpredictability in the art with regard to which xylanases would yield less sticky dough. Indeed, the fungal and bacterial xylanases had different effects on the stickiness of the dough produced in the specification. *See e.g.* Table 2 of the specification. Additionally, this data also shows that there are differences in the stickiness of dough produced by different bacterial xylanases. *See* Table 4.

Thus, Appellants maintain that a dough created with a bacterial xylanase which was less sticky than a dough made with a fungal xylanase was unexpected and is evidence of the non-obviousness of the present invention.

C. The Cited Art Teaches Away from the Claimed Invention.

The Examiner relies on Poutanen, discussed *supra*, as a primary reference in the present rejection. As discussed previously, the only reference to dough stickiness in Poutanen may be found in Table 3, on page 304. Table 3 discloses that a specific fungal xylanase from *Aspergillus*

caused an increase in the specific volume of wheat bread without causing stickiness citing Maat, also discussed *supra*:

> A specific *Aspergillus awamori* endo-xylanase was effective in increasing the specific volume of wheat bread without causing stickiness. Maat et al. (1991).

Poutanen at 304.

Appellants maintain that the Poutanen and Maat references teach *away* from the use of bacterial xylanases in bakery products or doughs for bakery products, as required by the claimed invention. Specifically Maat states that the use of *Aspergillus awamori* (fungal) xylanase is effective in "increasing the specific volume of breads, without giving rise to a negative side effect on dough handling (stickiness of the dough) *as can be observed with xylanases derived from other fungal or bacterial sources*." Maat at 349 (emphasis added).

The Examiner has maintained that he does not have to consider the disclosure in Maat as the reference was never cited by the Examiner and that the argument is not relevant to the Examiner's rejection:

> [The] examiner would like to state that said reference was never cited by the Examiner in the rejection and the applicants use of the reference in the argument is not relevant to the basis of [the] rejection adopted by the examiner.

Advisory Action at 9 and Final Office Action at 10-11 (text in brackets added). The Examiner further stated that the reference was not relevant as the Examiner had already provided the reason for the use of a xylanase isolated by Paice, Wolf, Campbell and Haarasilta:

> [The] examiner has provided the reason for the use of xylanases isolated by Paice et al., Wolf et al., Campbell et al., and Haarasilta et al. that teach the structural and functional elements of the instant invention and having the desired activity.

Advisory Action at 9 and Office Action at 11.

However, Appellants maintain that the Examiner must consider Maat as 1) the Examiner must consider the Poutanen reference as a whole; 2) Maat was cited in Table 3 by Poutanen for the very proposition the Examiner is relying upon in his rejection (*i.e.* "the addition of xylanase was effective in increasing the specific volume of wheat bread without causing stickiness (Table 3, page 304)" (Advisory Action at 3); and 3) the standard for an obviousness analysis is one of ordinary skill in the art, not the Examiner's perspective.

> To reach a proper determination under 35 U.S.C. 103, the examiner must step backward in time and into the shoes worn by the hypothetical "person of ordinary skill in the art" when the invention was unknown and just before it was made.

MPEP § 2142. An obviousness analysis requires what one of skill in the art would have understood the reference to teach. 35 U.S.C. § 103 ("A patent may not be obtained...if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.") (emphasis added).

Thus, Appellants maintain that one of ordinary skill in the art reviewing Poutanen would have considered that only the specific *Aspergillus* fungal xylanase cited in Table 3 of Poutanen, and relied upon by the Examiner in his rejection, was effective at increasing the specific volume of wheat bread without causing stickiness. On considering whether the teaching of Poutanen could be applied to other xylanases, a person of ordinary skill in the art would have sought out further information in the form of the reference cited by Poutanen, *i.e.*, Maat. Maat discloses that the specific fungal enzyme discussed in Poutanen is very effective at increasing specific volumes of breads without giving rise to dough stickiness as is observed with other xylanases derived from other fungal or bacterial sources:

> We have identified a particular β-(1,4)-xylanase produced by an Aspergillus niger var. awamori strain as being very effective in increasing specific volume of breads, without giving rise to a negative side effect on dough handling (stickiness of the dough) *as*

> *can be observed with xylanases derived from other fungal or bacterial sources.*

Maat at 349 (emphasis added).

The Examiner must rely upon the teaching of the cited art as a whole and how it would be interpreted by one of skill in the art. Maat is being cited by Appellants in order to rebut the Examiner's interpretation of Poutanen and provide support for Appellants' assertion that Poutanen teaches away from the use of bacterial xylanases. In view of the above, and because Maat is cited in Poutanen for the exact proposition the Examiner is relying upon in his rejection (*i.e.*, xylanases improve the stickiness of dough), the Examiner cannot just choose to ignore Maat.

Thus, Appellants maintain that the cited art teaches away from the use of bacterial xylanases as is presently claimed for decreasing dough stickiness.

2. Claim 67 is not obvious over the cited art.

The Examiner also rejected claim 67 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Haarasilta or Poutanen, Paice or Wolf and Campbell and further in view of Autio. Advisory Action at 5 and Final Office Action at 6. The Examiner states that the combination of Haarasilta or Poutanen, Paice or Wolf and Campbell do not specifically teach xylanase free of glucanase enzymes. *Id.* However, the Examiner alleges that Autio teaches "the effects of purified xylanase and glucanase on the structural and baking characteristics of doughs, said reference discloses that the addition of glucanase had a hardening effect on doughs and bakery products." Advisory Action at 5-6 and Final Office Action at 6-7. The Examiner alleges that it would have been obvious to combine the teachings of Haarasilta or Poutanen, Paice or Wolf, Campbell and Autio to produce the baking products and doughs with xylanase devoid of glucanase as the "presence of glucanases catalyzes the breakdown of substrates that results in unwanted hardening effect on said products." Advisory Action at 6 and Final Office Action at 7 .

For the reasons discussed above, Autio does not cure the deficiencies of Haarasilta Poutanen, Paice, Wolf and Campbell. Additionally, Autio relates to the use of a purified fungal (*Trichoderma reesei*) xylanase in rye doughs and the effects thereof. *See* Autio, Materials & Methods at 19. Autio does not disclose the use of a bacterial xylanase in dough, let alone a bacterial xylanase expressed from the nucleotide sequence of SEQ ID NO:6. In addition, Autio does not teach or suggest that the use of such a bacterial enzyme will give rise to a less sticky dough compared with an otherwise identical dough prepared by incorporating a fungal xylanase instead of a bacterial xylanase.

3. Conclusion

In view of the foregoing discussion, Appellants submit that the subject matter of claims 56-67 and 69-70 are not obvious over the cited references. The Examiner has failed to establish a *prima facie* case of obviousness, particularly without relying upon impermissible hindsight. Additionally, the cited art teaches away from the claimed invention and Appellants have provided evidence of non-obviousness in the form of unexpected results.

Accordingly, Appellants respectfully request that the Board reverse the Examiner's obviousness rejection and remand this application for issue.

Respectfully submitted,

Date March 27, 2009

By Julie Heider
AV
Michele M. Simkin
Attorney for Appellants
Registration No. 34,717
Reg # 54,161

FOLEY & LARDNER LLP
Customer Number: 22428
Telephone: (202) 672-5538
Facsimile: (202) 672-5399

VIII. CLAIMS APPENDIX

56. A bakery product or a dough for making a bakery product comprising a polypeptide expressed from the nucleotide sequence of SEQ ID NO:6, wherein said bakery product or dough for making a bakery product is suitable for use in a foodstuff.

57. The bakery product or dough for making a bakery product of claim 56, wherein said polypeptide does not contain a leader sequence.

58. The bakery product or dough for making a bakery product of claim 56, wherein said polypeptide has the amino acid sequence of SEQ ID NO:5.

59. The bakery product or dough for making a bakery product of claim 57, wherein said polypeptide has the amino acid sequence of amino acids 29-213 of SEQ ID NO:5.

60. A dough for making a bakery product prepared by incorporating a bacterial xylanase comprising a polypeptide expressed from the nucleotide sequence of SEQ ID NO:6, whereby the resultant dough is less sticky than an otherwise identical dough prepared by incorporating a fungal xylanase instead of said bacterial xylanase.

61. The dough of claim 60, wherein said polypeptide does not contain a leader sequence.

62. The dough of claim 60, wherein said polypeptide has the amino acid sequence of SEQ ID NO:5.

63. The dough of claim 62, wherein said polypeptide has the amino acid sequence of amino acids 29-213 of SEQ ID NO:5.

64. A bakery product prepared by baking the dough of claim 60.

65. The dough of claim 60, comprising wheat flour, water and a bacterial xylanase expressed from the nucleotide sequence of SEQ ID NO:6.

66. The dough of claim 65, wherein said bacterial xylanase is from a *Bacillus subtilis* strain.

67. The dough of claim 65, wherein said bacterial xylanase is free of detrimental levels of glucanase enzymes.

69. The dough of claim 65, further comprising yeast.

70. A bakery product prepared by baking the dough of claim 69.

IX. EVIDENCE APPENDIX

1. Maat *et al.*, "Xylanases and their application in bakery," in XYLANS & XYLANASES ed. J. Visser *et al.* Elsevier: 349-360 (1992), cited by Appellants as document **A27** in the IDS filed August 18, 2003 and considered by the Examiner May 11, 2006.

X. RELATED PROCEEDINGS APPENDIX

None

Xylans and Xylanases, edited by J. Visser et al.

39
, de Gruiter

'3), pp 30 -

273 -281

118 -121

dustrial

ion No 0371

No 803/90,

1990
Knowles, in
),

wish to

245

Xylanases and their application in bakery

J. Maat, M. Roza, J. Verbakel, H. Stam, M.J. Santos da Silva, M. Bosse, M.R. Egmond, M.L.D. Hagemans[a]
R.F.M. v. Gorcom, J.G.M. Hessing, C.A.M.J.J.v.d. Hondel, C. v. Rotterdam[b] .

[a] Unilever Research Laboratorium, Olivier van Noortlaan 120, Vlaardingen, The Netherlands
[b] Medisch Biologisch Laboratorium-TNO, Lange Kleiweg 137-139, Rijswijk, The Netherlands

Abstract

In order to improve dough handling and the quality of the final baked product, especially of products made of lower quality wheat flour, it is common practice to add a number of ingredients. Especially enzymes play an important role in this process.

We have identified a particular ß-(1,4)-xylanase produced by an Aspergillus niger var. awamori strain as being very effective in increasing the specific volume of breads, without giving rise to a negative side effect on dough handling (stickiness of the dough) as can be observed with xylanases derived from other fungal or bacterial sources.

The gene encoding the xylanase has been cloned and fully characterized by DNA sequence analysis. Based on this analysis , the xylanase is produced as a 211 amino acid residues long precursor, which is converted post-translationally into a 184 aa. residues long mature protein. The DNA derived protein sequence shows a considerable homology (ca. 50%) with xylanases from B.circulans, B.pumilis and C.acetobutylicum.

The production of the xylanase could be enhanced twenty- to thirty-fold upon transformation of the original A.niger var. awamori strain with multiple copies of the chromosomal gene using amidase S as selection marker. This "cloned" enzyme is fully identical to the "original" enzyme, and has identical enzymological and application properties.

INTRODUCTION

Flour from cereals, water, yeast and salt are the basic ingredients of bread and other baked foods. For decades it has been common practice to add ingredients that have a positive effect on the processing of the dough and/or the quality of the baked products. Process criteria are especially related to dough handling (machinability) and process yield (water retention, increased or decreased proofing times). The product quality is mostly evaluated on the basis of appearance (volume, colour) and eating properties (crumb elasticity, flavour).

The volume of the bread is considered one of the most important parameters as it has direct implications for eating properties. Various bread improver components as emulsifiers (CSL; calcium stearoyllactylate), oxidising agents (bromate, [dehydro]-ascorbic acid) and enzymes from plant (barley or wheat malt) or microbial (fungal) origin have been shown to positively influence the bread volume (1) especially when lower quality flours with a low gluten content are being used.

The commercial enzyme preparations combine several enzyme activities, and vary considerably in composition and ratio of these activities, depending on the source.
In order to better control the effect of the enzymes with respect to the above mentioned process and product criteria we have embarked on the analysis of fungal enzyme preparations. This has eventually resulted in the identification of xylanase as a very important contributor to improved bread volume and design of a production route of a specific xylanase, involving recombinant DNA procedures.

MATERIALS AND METHODS

Enzyme preparations for analysis of bakery application were obtained from various commercial suppliers: Grindstedt, Amano, Quest Biocon Int., Alko, Cultor etc. or by in-house production from various fungal strains (see below).

Assay of enzyme activities

Overall xylanase and alpha-amylase activity were determined by measuring the production of newly liberated reducing groups using the DNS-method (2). 1 ml of a 1% solution (suspension) of (oat spelt) xylan or starch in 0.05 mol./l acetate buffer (pH 5.5) were incubated at 40°C for 30 minutes with such an amount of enzyme to produce an optical density at 543 nm of 0.5 - 1.0, after reaction with the reagent. Incubated samples were further analyzed as described in the referred method. A unit of xylanase activity then is defined as the amount of enzyme which, per minute, releases an amount of reducing groups from xylan equivalent to 1 mg xylose.

Thin layer chromatography of sugars

T.L.C. of oligosaccharides was performed as described by Lombard (3).

Fractionation of flour; isolation of starch tailings

Starch tailings can be isolated from wheat flour by washing a (water-flour) dough with 0.1M NaCl. After sieving the gluten the resulting slurry can be centrifuged. On the fast sedimenting fraction, which mainly consists of starch, a "lighter" brown-coloured layer can be observed, which is tentatively called the "starch-tailings" and which can be further purified by washing and centrifugation. The resulting fraction consists of starch and insoluble pentosans. The water fraction of the first sedimentation contains the water-soluble pentosans and proteins (globulins, albumins).

In order to obtain large amounts of spores, spores from the second streak were streaked through onto plates with rich medium and incubated for 5-6 days at 25-28°C. The resulting spores were stored as a suspension (10^8-10^9 spores/ml) or adsorbed to silica gel so that the spores can be stored for a long time.

RESULTS AND DISCUSSION

The importance of xylanase for flour/bread improvement was revealed by the following experimental procedures :

Influence of starch tailings on the bread volume

Figure 1 shows the effect of variation in the amount of starch tailings added to a reconstituted dough system. The amount of water added to each dough was adjusted to the amount of tailings added. Clearly reduction of the amount of starch tailings has a positive effect on the volume of the micro-loaves. The insoluble pentosans present in the tailings are responsible for this effect as starch, the other component of starch tailings, as such did not show this effect (results not shown).

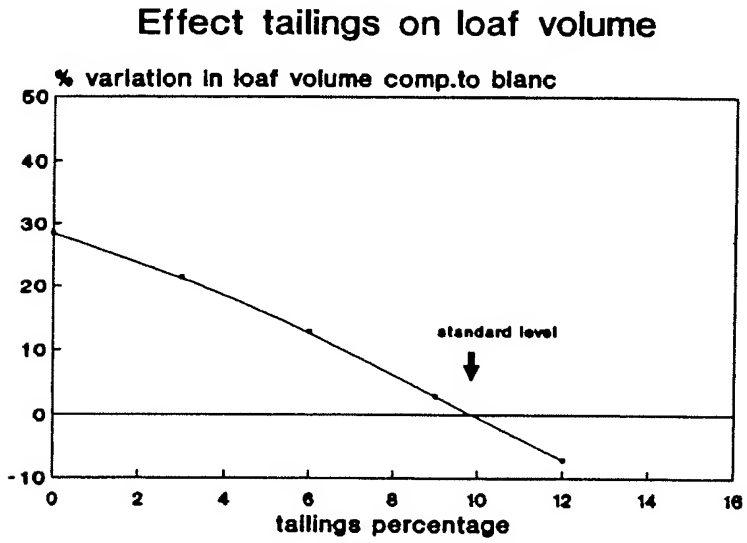

Figure 1. Variation in loaf volume as a function of wheat starch tailings added to a reconstituted dough. Loaf volume of control (10% level) is 35 ml.

Baking tests

Micro-loaves with and without added enzymes were prepared according to Shogren (4). (Specific) volumes were determined using the seed displacement technique. Flours used are indicated in the legend of the figures.

Strains and plasmids

Aspergillus niger var. awamori (CBS 115.52) was obtained from the Centraal Bureau Schimmelcultures (Baarn, The Netherlands).

Escherischia coli strains JM109 and NM539, used for plasmid isolation and construction of a gene bank, were obtained from Promega Biotec.

Protein analysis and characterisation

Xylanase can be purified by chromatographic procedures ; typically ion-exchange chromatography on DiEthyl Amino-Ethyl (DEAE-) Sepharose, following elution at various NaCl concentrations (see Fig.2) and gel filtration using Ultrogel AcA-54.

Aminoterminal amino acid sequence residues were determined using an Applied Biosystems 470A pulsed liquid phase protein sequencer.

Molecular biological procedures

DNA isolation, manipulation and cloning procedures were used essentially as described by Maniatis et al. (6). DNA sequence analysis was performed using the dideoxy-chain terminator procedure according to Sanger et al. (7). Synthetic DNA was prepared using the Applied Biosystems DNA synthesizer.

Culturing conditions

Media : xylan medium contains 1% xylan, 0.56% yeast extract with amino acids (Difco) and 0.1% cas. amino acids. Medium with wheat bran consists of 4 g wheat bran in 50 ml mains water, to which 50 ml of a salt solution (pH 5.0) is added up to a final concentration of 0.5% $(NH_4)_2SO_4$, 0.15% KH_2PO_4, 0.025% $MgSO_4$ and 0.025% KCl. Rich medium for expression tests is minimal medium (0.05% $MgSO_4$, 0.6% $NaNO_3$, 0.05% KCl, 0.15% KH_2PO_4 and trace elements), with 1% glucose, 0.2% trypticase (BBL), 0.5% yeast extract, 0.1% cas. amino acids and vitamin. Starch medium contains 5% starch and 0.1% glucose in minimal medium. Media were sterilised for 30 minutes at 120°C. Medium (100 ml in a 500 ml flask) was inoculated with 2×10^5 spores/ml, followed by culturing in an air incubator (300 rpm) at 25°C for different periods. Cultures with wheat bran as an inducer were inoculated with 4×10^5 spores/ml.

Aspergillus transformation

Aspergillus niger var. awamori protoplasts were made from mycelium by means of Novozym 234 (NOVO). The yield of protoplasts was $1\text{-}5 \times 10^7$/g mycelium and the viability was 3-8%. Per transformation $3\text{-}8 \times 10^5$ viable protoplasts were incubated with 5, 10 or 20 µg plasmid DNA that had been subjected twice to CsCl purification. Transformed protoplasts were plated on osmotically stabilised selection plates (acetamide as a nitrogen source) and incubated at 25°C. After 6-10 days colonies were visible.

Isolation and characterisation of xylanase

Enzyme preparations of fungal origin (Aspergillus, Trichoderma) contain a substantial variation of enzyme activities (amylases, cellulases, endo- and exo-xylanases, arabinases, proteases etc.) Not only the type but also their respective quantities vary from one preparation to another, which hampers the application of these preparations in the bakery. Upon chromatographic separation by, for example, ion exchange chromatography, enzyme fractions can be obtained which have a varying effect on bread volume improvement.

Figure 2 shows a typical example of a commercial enzyme preparation, in this case obtained from Grindsted (Denmark).

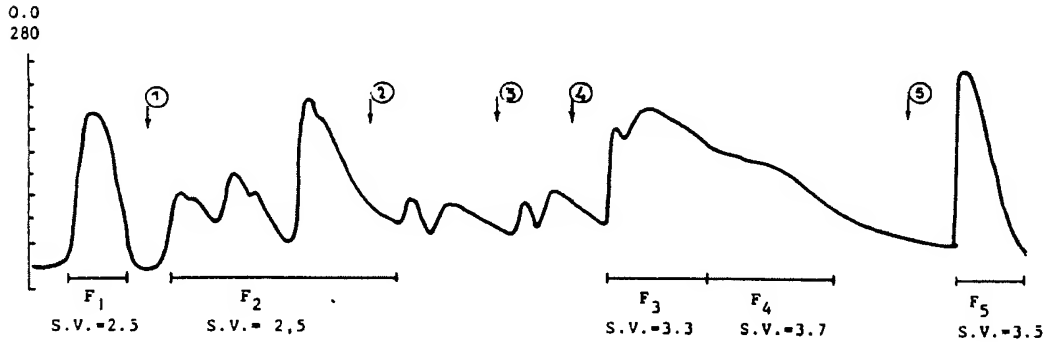

Figure 2. Separation of Grindamyl on DEAE-Sepharose (0.05 mol/l acetate buffer pH 5.5)
S.V. = Specific Volume of microloaf baked with addition of 50 µl of each fraction.
Column development with respectively:
1 = 0.05 mol/l NaCl; 2 = 0.075 mol/l NaCl; 4 = 0.1 mol/l NaCl;
4 = 0.15 mol/l NaCl; 5 = 0.3 mol/l NaCl

Analysis of the enzyme activity by (oligo)saccharide products produced from (starch-free) wheat starch tailing is shown in Fig.3.

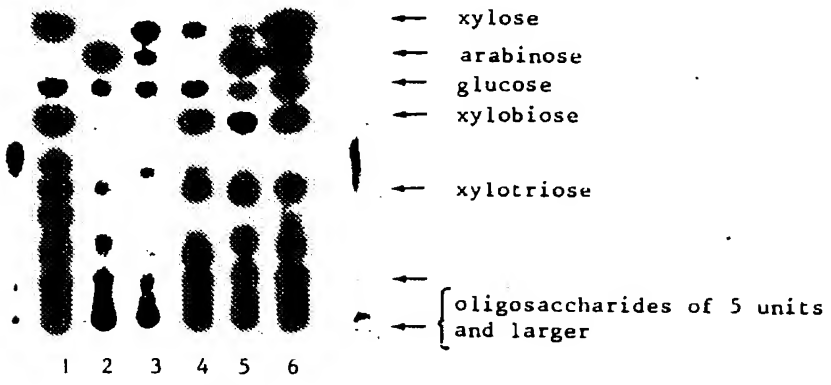

Figure 3. Thin layer chromatography soluble saccharides after incubation of (starch-free) starch tailings, with respectively:
1 = fraction F^1 (Figure 2)
2 = F^2 etc.; 6 = overall activity before separation

Fractions 1, 4 and 5 show the production of larger oligo-saccharides in addition to small oligosaccharides and consequently can be regarded as to contain endo-xylanase activity. Fraction 2 clearly contains alpha-*L*-arabinosidase activity, while fraction 5 contains this activity in addition to endo-xylanase activity. The majority of alpha-amylase and xylobiase (beta-1,4-xylosidase) activity is present in fraction 3.
All fractions were tested in the baking experiments and fraction 4 clearly had the highest positive effect on loaf volume.

Xylanase fraction 4 was further purified into two fractions by gel filtration on Ultrogel AcA-54. The top fraction of each peak was nearly pure when tested by iso-electric focusing. The top fraction contained alpha-amylase as a single activity, while the top fraction of the second peak contained only xylanase activity. Xylanase and alpha-amylase have very similar isoelectric points (pI's); the molecular weights however differ by a factor 2, as can be seen in Fig. 4.

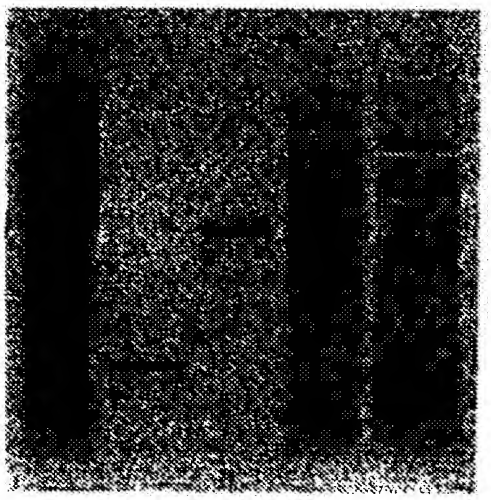

Figure 4. S.D.S.-polyacrylamide gelelectrophoresis pattern:
Lane 1: commercial preparation A; 2: purified xylanase;
3: purified α-amylase; commercial preparation B;
5: molecular weight markers are 90, 67, 43, 30, 20.1
and 14.4 KD respectively from top to bottom

Baking experiments with the individual and combined fractions showed that this xylanase is mainly responsible for increased loaf volume (see also below).
The purification and characterisation of a similar xylanase of pI 3.65 has been described by Fournier et al.(8)

small
ty. Fraction
in addition
ase) activity

est positive

gel AcA-54.
g. The top
econd peak
ctric points

lanase is

escribed by

Cloning and characterisation of the xylanase gene

From a screening of xylanase producers (performed at the TNO-CIVO Institute Zeist by W. Knol) of fungal origin, an Aspergillus niger var. awamori was selected. This strain produced the correct type of xylanase in relatively high amounts as compared to other xylanases.

Xylanase of pI 3.7 was purified to homogeneity from a A. niger var awamori culture grown on wheat bran, using procedures similar to the one described above.

Amino acid sequence analysis of its amino terminus by the automated Edman degradation yielded a sequence of 17 residues. Based on this sequence 4 synthetic DNA probe mixtures were synthesized. The Xylo6 probe as shown in Fig.5 gave a good hybridisation on Southern blots of genomic DNA digested with several enzymes under rather stringent conditions of hybridisation and filter washing.

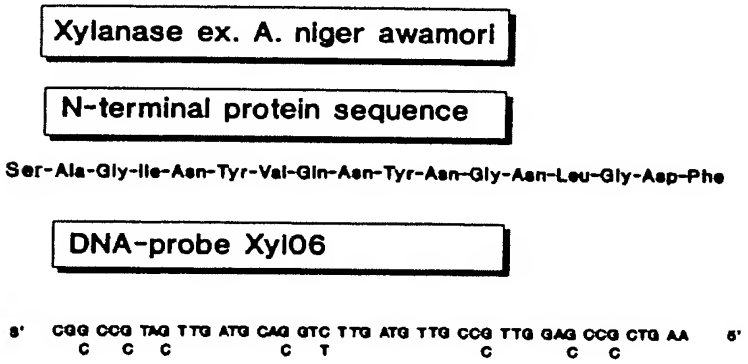

Figure 5. NH_2-terminal amino acid sequence (pI 3.7) of A.niger awamori; The sequence extending from Ala (2) was used for devising a 47 mer DNA probe with variations at the indicated positions. Hybridisation of radioactive probes took place in 6x Standard Saline Citrate (SSC) at 56°C. Filters were washed in 3x SSC at 50°C.

This Xylo6 probe was subsequently used to screen a genomic DNA library of A. niger var. awamori made in lambda EMBL3 phage. Of the 65.000 plaques screened (32 x the genome), three positively hybridizing phages were isolated and purified. 2.2 kb Pst I-Pst I DNA fragments, which strongly hybridize with Xylo6 from two of the phages were subsequently subcloned and fully characterised side by side by DNA sequence analysis.
The DNA sequence of the pre(pro)xylanase gene (xylA) is shown in Fig.6. The mature xylanase protein is preceded by a leader peptide of 27 amino acids. Between the alanine residues at the positions 16 and 17 a cleaving site is probably present for the signal peptidase. From the length of the leader peptide it can be derived that a second processing site is present in the protein. The cleaving of the bond between Arg 27 and Ser (28) possibly takes places by a KEX2-like protease.

In the xylA gene an intron of 49 or 76 bp (231-279 or 231-306) was predicted on the basis of the presence of sequences corresponding to "donor" and "acceptor" sites of introns in aspergilli. Definite proof of the absence of a 76 bp intron was obtained by the isolation of a xylanase peptide, generated by Staphylococcus aureus protease digestion, with the sequence Tyr-Ser-Ala-Ser-Gly-.... . This peptide can only be localised in the protein from position 302 in the DNA. The DNA-derived protein sequence reveals the xylanase protein to have 184 amino acid residues, with a corresponding molecular weight of 19890 D and a calculated pI of 3.692.

```
                  .        20        .        40        .        60
ATGAAGGTCACTGCGGCTTTTGCAGGTCTTTTGGTCACGGCATTCGCCGCTCCTGTGCCG
---------+---------+---------+---------+---------+---------+
M  K  V  T  A  A  F  A  G  L  L  V  T  A  F  A  A  P  V  P

                  .        80        .       100        .       120
GAACCTGTTCTGGTGTCGCGAAGTGCTGGTATTAACTACGTGCAAAACTACAACGGCAAC
---------+---------+---------+---------+---------+---------+
E  P  V  L  V  S  R  S  A  G  I  N  Y  V  Q  N  Y  N  G  N
                     |=> mature xylanase

                  .       140        .       160        .       180
CTTGGTGATTTCACCTATGACGAGAGTGCCGGAACATTTTCCATGTACTGGGAAGATGGA
---------+---------+---------+---------+---------+---------+
L  G  D  F  T  Y  D  E  S  A  G  T  F  S  M  Y  W  E  D  G

                  .       200        .       220        .       240
GTGAGCTCCGACTTTGTCGTTGGTCTGGGCTGGACCACTGGTTCTTCTAAGTGAGTGACT
---------+---------+---------+---------+---------+---------+
V  S  S  D  F  V  V  G  L  G  W  T  T  G  S  S  N

                  .       260        .       280        .       300
GTATTCTTTAACCAAAGTCTAGGATCTAACGTTTTCTAGCGCTATCACCTACTCTGCCGA
---------+---------+---------+---------+---------+---------+
                                          A  I  T  Y  S  A  E

                  .       320        .       340        .       360
ATACAGTGCTTCTGGCTCCTCTTCCTACCTCGCTGTGTACGGCTGGGTCAACTATCCTCA
---------+---------+---------+---------+---------+---------+
 Y  S  A  S  G  S  S  S  Y  L  A  V  Y  G  W  V  N  Y  P  Q

                  .       380        .       400        .       420
GGCTGAATACTACATCGTCGAGGATTACGGTGATTACAACCCTTGCAGCTCGGCCACAAG
---------+---------+---------+---------+---------+---------+
 A  E  Y  Y  I  V  E  D  Y  G  D  Y  N  P  C  S  S  A  T  S

                  .       440        .       460        .       480
CCTTGGTACCGTGTACTCTGATGGAAGCACCTACCAAGTCTGCACCGACACTCGAACTAA
---------+---------+---------+---------+---------+---------+
 L  G  T  V  Y  S  D  G  S  T  Y  Q  V  C  T  D  T  R  T  N

                  .       500        .       520        .       540
CGAACCGTCCATCACGGGAACAAGCACGTTCACGCAGTACTTCTCCGTTCGAGAGAGCAC
---------+---------+---------+---------+---------+---------+
 E  P  S  I  T  G  T  S  T  F  T  Q  Y  F  S  V  R  E  S  T

                  .       560        .       580        .       600
GCGCACATCTGGAACGGTGACTGTTGCCAACCATTTCAACTTCTGGGCGCAGCATGGGTT
---------+---------+---------+---------+---------+---------+
 R  T  S  G  T  V  T  V  A  N  H  F  N  F  W  A  Q  H  G  F

                  .       620        .       640        .       660
CGGAAATAGCGACTTCAATTATCAGGTCATGGCAGTGGAAGCATGGAGCGGTGCTGGCAG
---------+---------+---------+---------+---------+---------+
 G  N  S  D  F  N  Y  Q  V  M  A  V  E  A  W  S  G  A  G  S

                  .       680
CGCCAGTGTCACGATCTCCTCTTAA
---------+---------+-----
 A  S  V  T  I  S  S  *
```

Figure 6. DNA sequence of the preproxylanase gene of A.niger var awamori

basis of
spergilli.
xylanase
Ser-Ala-
DNA
residues,

The xylanase protein shows a considerable homology to the xylanases of B. pumilis (9), B.circulans (10) and Clostridium acetobutylicum (11) as can be seen in Fig.7

```
(1)                  S A G I N Y V Q N Y N G N L G D F T Y D E        AA
(1)                        A S T D   W Q   W T   G G G I V        BC
(1)      R T I T N N E M   N H S G Y D   E L - W K   Y G N T S      BP
(32)     K T I T S N E I   V   G G Y D   E L - W K   Y G N T S      CA
(1)        S G T P S S T   T D G G Y Y   S W - W T   G A G D A      SC

(22)     - - - - - S A G T F S M Y W E D G V S S D F V V C L        AA
(17)   N A V N G   G   N Y   V N   S N T G N - -         K          BC
(27)   M T L N - N G   A     A G   N N I G N A L   R K   -          BP
(50)   M T L K - N G   A     C Q   S N I G N A L   R K   -          CA
(26)   T Y #                                                       SC

(43) - - - - - G W T T G S S K - - A I T Y S A E Y S A S - G S S    AA
(41) - - - - -             P - F R T   N   N   G V W   P N   N -    BC
(51) K K F D S T R   H H Q L G N I S   N   N   - S F N P S   N -    BP
(82) K K F N D T Q   Y K Q L G N I S V N   D C - N Y Q P Y   N -    CA

(65) S Y L A V Y G W V N Y P Q A E Y Y I V E D Y G D Y N P C S      AA
(64) G     T L       T R S   L I       V V D S W   T   R   T G    BC
(79)       C         T Q S   L             D S W   T   R   T G    BP
(110)      C         T S S   L V           D S W   S W R   P G    CA

(94)  S A T S L G T V Y S D G S T Y Q V C T D T R T N E P S I      AA
(93)  - -   Y K       K       G     D I Y   T     Y   A            BC
(108) - - A Y K   S F   A     G     D I Y E T     V   Q            BP
(139) - G     K     I T V     G I   D I Y E T     I   Q            CA

(122) T G - T S T F T Q Y F S V R E S T R T S G T - - -            AA
(119) D   D R T             W       Q   K   P T G S N A T        BC
(134) I   I A T -   K       W       Q T K           - - -        BP
(166) Q   N - T     K       W       R T K           - - -        CA

(143) V T V A N H F N F W A Q H G F G N - S D F N Y Q - V M A V E  AA
(144) I   F T     V   A   K S     M N L G   N W A     -       T    BC
(155)   S   S A     R K   E S L   M P M G - - K M   E T A F T      BP
(187) I S   S K     A A   E S K   M P L G - - K M H E T A F N I    CA

(171) A W S G A G S A S V T I S S *                                AA
(173) G Y Q S S     S N     V W *                                  BC
(183) G Y Q S S       N   M T N Q L F I G N *                      BP
(215) G Y Q S S   K   D   N S M   I N I G K *                      CA
```

Figure 7. Comparison of amino acid sequences of xylanases from A.niger awamori (AA), B.circulans (BC), B.pumilis (BP), C.acetobutylicum (CA) and S.chizophyllum commune (SE). Only amino residues non-identical to A.niger awamori are indicated.

A very substantial homology regarding the first 45 amino acid residues of the xylanase from Aureobasidium pullulans (12) is also observed (results not shown).

Expression of the xylanase gene

Three expression vectors were constructed containing the genomic xylA-gene from the translation start including the xylA transcription terminator.

1. pAW14S comprises a 5.3 kb SalI-SalI-chromosomal DNA fragment from A. niger var awamori on which the xylA-gene is located with its own expression signals (xylA-promoter). Further a 5.3 kb fragment of A. nidulans on which the acetamidase (amdS) gene is located, is present on this plasmid. The cloning vehicle is based on the pUC19 E.coli-vector.
2. pAW14S-2 differs from pAW14S in that the A. niger var awamori fragment located upstream of the ATG codon of the xylA-gene is replaced by the constitutive expression signals (up to the ATG triplet) of the A. nidulans glyceraldehyde-3-phosphate dehydrogenase (gpdA) gene. The right connection between the gpdA promoter and the ATG codon of the xylA-gene was obtained by means of a synthetic DNA fragment.
3. pAW14S-3 comprises the inducible expression signals of the A. niger var niger glucoamylase (glaA) gene up to the ATG codon followed by the xylA-gene and terminator sequences as in pAW14S-2.

The A. niger var awamori strain was transformed with the expression vectors according to the procedure described in the Materials and Methods section. A frequency of 0.03 to 0.23 transformants per µg vector DNA was obtained. Transformants containing multiple copies of the vector were selected on the basis of their xylanase production.

Typically the transformants produced the xylanase activities as given in Table I upon growth on several media using shaking flasks.

Table I
Expression of xylanase by wild type and transformed A. niger var awamori.
Expression values are in 10^3U

Transformed plasmid	Medium			
	xylan	"rich"	starch	wheat bran
-	3	-	-	3
pAW14S	60	_	-	78
pAW14S-2	-	20	-	20
pAW14S-3	-	-	60	35

Especially the expression of the xylanase gene under its own promoter on growth on wheat bran medium was quite high, i.e. enhanced 20-30 times more than the wild type expression. This production could be further increased upon fermentation optimisation (results not shown).

Application of xylanase

The effect of addition of xylanase (at 240 U/mg) at various levels on the specific volume of 50 gram rolls made from Banket flour using a lean recipe is shown in Fig. 8.

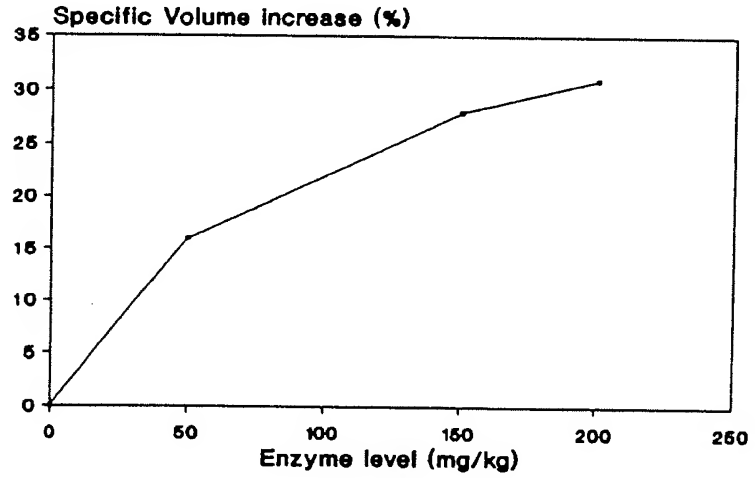

Figure 8. Relevative increase in specific volume (ml/g) as a function of xylanase enzyme to Banket flour dough. The xylanase concentration is 250 U/mg

The same effect can be established if, in addition, other bread improving ingredients such as fat, vitamin C, emulsifiers and alpha-amylase are added.

Figure 9 gives an example of micro-loaves baked with 0.2 mg/kg (pure) alpha-amylase, 0.2 mg/kg (pure) xylanase, and a combination of the two. Clearly an additive effect can be observed. Fine tuning of the ratio and the total amount depends on the flour used, the dough preparation process, other ingredients and the type of product to be produced.

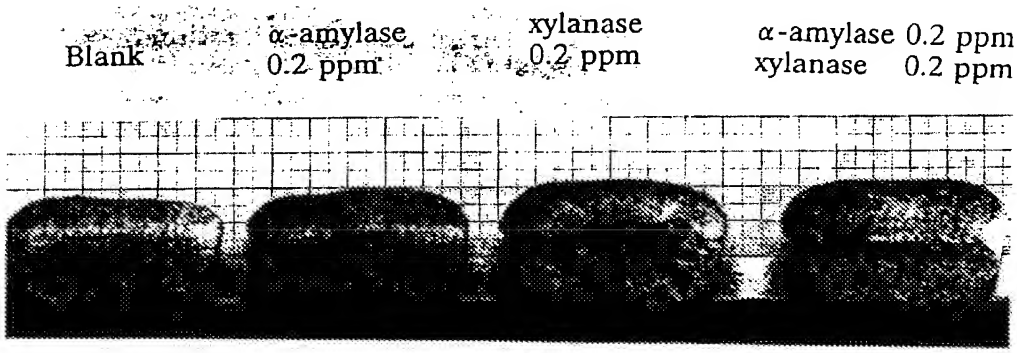

Figure 9. Micro-loaves (10 g) made with Banket flour. Amounts of α-amylase and xylanase are indicated.

Moreover, the xylanase enzyme described here had a good effect on the crumb structure and gave rise to little or no dough stickiness contrary to preparations of Trichoderma viridi or T. reesei origin which showed much dough stickiness.

Working hypothesis

The effect of xylanase on bread volume improvement can be ascribed to redistribution of water from the pentosan phase to the gluten (continuous) phase. The volume fractions of both are 10 and 17% respectively in a dough prepared from a regular wheat flour containing ca. 2-3% pentosans and 12% gluten. The increase in gluten volume fraction gives the gluten more extensibility, which eventually results in a better ovenspring.

REFERENCES

1. Kruger J.E., Lineback D., Stauffer, C.E. eds., American Association Cereal Chemistry Inc. St. Paul, U.S.A., Enzymes and their role in cereal technology.
2. Amado, R., Neukom, H., News approaches to research on cereal carbohydrates (1985), 241 Elsevier Science Publishers, Amsterdam, The Netherlands.
3. Lombard, A., Tourn, M.L., Bulfra, M., J. of Chromatography, 184 (1977) 242
4. Shogren, M.D., Finney, K.F., Cereal Chemistry 61 (1984) 418
5. Wernars, K., DNA-mediated transformation of the filamentous fungus Aspergillus nidulans, Thesis, Agricultural University of Wageningen, 1986
6. Maniatis, T., Fritsch, E.F., Sambrook, J., (1982), Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory.
7. Sanger, F., Nicklen, S., Coulson, A.R., Proc. Natl. Acad. Sci. USA 74 (1977) 5462.
8. Fournier, R.A., Frederick, M.M., Frerick, J.R., and Reilly, P.J. Biotechn. Bioeng, 27 (1985), 596
9. Fukusaki, E., Panbangred, W., Shinmyo, A., and Okada, H., FEBS Letters, 171 (1984) 197
10. Yang, R.C., MacKenzie, C.R., Narang, S.A., Nucleic Acid Res., 16 (1988) 7187
11. Zappe, H., Jones, W.A. and Woods, D.R., Nucleic Acids Res., 18 (1990) 2179
12. Leathers, T.D., Biotechn. Letters 10 (1988) 795